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Correlation Between Connexin 32 Gene Mutations and Clinical Phenotype in X-Linked Dominant Charcot-Marie-Tooth Neuropathy

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We studied the relationship between the genotype and clinical phenotype in 27 families with dominant X-linked Charcot-Marie-Tooth (CMTX1) neuropathy. Twenty-two families showed mutations in the coding region of the connexin32 (cx32) gene. The mutations include four nonsense mutations, eight missense mutations, two medium size deletions, and one insertion. Most missense mutations showed a mild clinical phenotype (five out of eight), whereas all nonsense mutations, the larger of the two deletions, and the insertion that produced frameshifts showed severe phenotypes. Five CMTX1 families with mild clinical phenotype showed no point mutations of the cx32 gene coding region. Three of these families showed positive genetic linkage with the markers of the Xq13.1 region. The genetic linkage of the remaining two families could not be evaluated because of their small size.

KEY WORDS: Charcot - Marie - Tooth neuropathy, connexin 32 gene, mutations

INTRODUCTION

One of Charcot's major contributions to neurology was the study he and Pierre Marie published on peroneal muscular atrophy based on the clinical study of five children with the disease [Charcot and Marie, 1886]. Howard Henry Tooth [1886] described in his Cambridge dissertation the same disease under the name "peroneal progressive muscular atrophy." It became obvious during the last 27 years of electrophysiological and pathological research that the single entity described in 1886 by Charcot, Marie, and Tooth should be reclassified into two major types. Consequently Charcot-Marie-Tooth disease, also known as hereditary motor and sensory neuropathy (HMSN) [Dyck and Lambert, 1968; Dyck et al., 1993], was classified into demyelinating (CMT1) and axonal (CMT2) forms. Molecular genetic studies further divided each of these two types of CMT into several genetic conditions which have a common phenotype [Ionasescu, 1995a]. The inheritance of CMT may be autosomal dominant with a frequency of about 76% of all cases [Ionasescu, 1995a] or autosomal recessive (unknown frequency in the USA) and X-linked (dominant CMTX1 or recessive CMTX2 and CMTX3) with a combined frequency of around 15%. The distinctive clinical criteria for X-linked CMT are no male-to-male transmission and affected males have more severe weakness than affected females. The CMTX1 gene has been localized by genetic linkage analysis on the proximal long arm of the X chromosome at Xq13.1 [Gal et al., 1985; Beckett et al., 1986; Fischbeck et al., 1986; Goonewardena et al., 1988; Ionasescu et al., 1988, 1992; Haites et al., 1989; Mostacciuolo et al., 1991; Bergoffen et al., 1993a].

An international consortium to which we belong has analyzed the genetic linkage data provided by 1139 genotyped individuals with CMTX1, including our 197 patients [Pericak-Vance et al., 1995]. The following markers were tested: AR (androgen receptor), PGK1P1, DXS106, DXS453, DXS227, DXS441, DXS56, and DXYS1X. The maximum lod score by two point analysis was $Z = 64.59$ at $\theta = 0$ for the DXS453 marker. No recombinants were seen for DXS453 although it was genotyped in all families. Multipoint analysis demonstrated a peak lod score of 101.92 at 0 recombination with the DXS453 locus. The genetic map used in the multipoint analyses established the following order of the markers separated by the genetic distances: AR-3.6 cM-DXS106-2.2 cM-DXS453-2.7 cM-DXS441-3.7 cM-DXYS1X. The studies of the CMTX1 consortium narrowed the region of interest to an approximately 1 cM region surrounding DXS453. For families where linkage to markers on chromosome X was inconclusive, i.e., the lod score was <3, additional linkage was per-

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formed with markers on chromosomes 17 and 1 where loci for CMT1A and CMT1B have been found, respectively. The results of these tests were negative (data not shown). The CMTX1 gene codes for connexin 32 and is at Xq13.1.

Recently Bergoffen et al. [1993b], Ionasescu et al. [1994], and Fairweather et al. [1994] defined mutations of the connexin 32 gene (cx32) that cosegregate with the CMTX1 phenotype in several families. The objective of this study was to determine if the types and locations of mutations of the cx32 gene have a significant influence on the clinical phenotype of CMTX1.

MATERIAL AND METHODS

The data presented includes 27 X-linked dominant CMT families with 197 patients from a pool of 171 previously identified CMT families with 813 affected members. Genomic DNA was isolated from leukocytes by the method of Kunkel et al. [1982] and digested with the restriction enzyme specific to the mutation in each family (Dde I, Hpa II, Hae III, or Bfa I). The DNA fragments were separated on agarose gels and visualized by staining with ethidium bromide.

Protocol for Clinical Examination/Evaluation

The protocol for clinical evaluation of all CMT patients included establishing the age of onset, the duration of the disease, the progressivity of the disease, the pedigree, a description of physical symptoms, such as pes cavus, hammertoes, scoliosis, abnormal gait, weakness of feet and hands, presence of Gowers and Trendelenburg signs, absence of deep tendon reflexes, sensory evaluation, coordination, balance evaluation, Romberg sign, evaluation of cranial nerves, breathing problems, the use of braces, surgery, and electrical tests (MNCVs and EMG).

Linkage Analysis

Two-point and multipoint linkage were performed first, using the MLINK and LINKMAP programs of the LINKAGE package (version 5.1) as described by Lathrop et al. [1984]. Equal male and female recombination rates were assumed. Multipoint lod scores were defined as the log 10 difference between the disease locus at a specific recombination fraction (θ = 0.00 to 0.49) from the test locus and the disease locus placed in an unlinked state (θ = 0.50). Log_{10} differences greater than or equal to 3 are taken as significant evidence for linkage (same as $>10^3$, 1,000:1 odds). Values between -2 and 3 are indecisive and values equal to or below -2 indicate exclusion from that region. The 95% confidence limits were defined as the θ values at which the lod score equaled the maximum lod score minus 1.

DNA Analysis

The cx32 gene coding region was amplified by the polymerase chain reaction (PCR). The PCR products were separated on 1.2% agarose gels, cut out with a razor blade, and then extracted with phenol/chloroform and precipitated with ethanol. DNA sequence analysis was performed with the SequiTherm cycle sequencing kit of Epicentre Technologies (Madison, WI) and 8% sequencing gels as previously reported [Ionasescu et al., 1994]. All 27 families were also tested for duplication of chromosome 17p11.2–p13 as previously described [Ionasescu et al., 1995a].

RESULTS

Clinical Findings

The 27 families displayed a mode of inheritance characteristic of an X-linked disorder, with no male-to-male transmission. Some of these families were previously described [Ionasescu, 1995a,b]. The diagnosis of CMT was based on established clinical criteria: distal muscle weakness, atrophy, areflexia, and foot deformities (pes cavus and hammer toes) present in 90% of cases. The onset of the disease occurred between 2 and 24 years of age with the exception of family 17, carrying a 29 base pairs deletion, in which the onset was congenital in four members, including one mother and her son. The weakness always started in the lower leg muscles, with stumbling and steppage gait. There was a slow progression with spreading of the weakness to the hands over a period of 5 to 10 years. Most of the patients showed also balance difficulty with positive Romberg sign. Distal sensory loss with glove and stockings distribution (touch, proprioception, and pallesthesia) was also present in 70% of cases. Scoliosis was present in 20% of the cases. The affected males had a more severe phenotype than the affected females. We distinguished three CMT phenotypes. The mild phenotype is accompanied by relative good muscle strength, compatible with normal gait and climbing stairs. The patients showed foot deformities and areflexia. The moderate CMT phenotype is characterized by weakness of the tibialis anterior and peroneal muscles requiring ankle foot orthosis (braces), positive Romberg sign, distal hypesthesia, weakness of palmar and dorsal interossei, and scoliosis. The severe CMT phenotype shows also some proximal limb weakness in addition to distal weakness. The patients need canes or wheelchairs. In addition, the sensory and balance impairment create additional handicaps for the patient. Breathing difficulty due to phrenic nerve involvement is sometimes present.

Electrophysiological Findings

Slowing of motor nerve conduction velocity (MNCV) was present in 25 of our 27 CMTX1 families. The average values were 20–30 m/sec, consistent with moderate demyelination of the screened nerves (peroneal, ulnar, and median). Prolonged terminal latencies (6–7 msec) were also present. The electromyogram (EMG) was usually normal in the cases with a demyelinating pattern. Two families showed normal MNCVs and abnormal EMG with fibrillation potentials, suggesting axonal neuropathy. Both families are among the five with positive linkage and no coding region mutations of connexin 32.

Molecular Genetic Results

The following types of mutations of the cx32 gene were identified: four nonsense mutations, eight missense mutations, two medium size deletions of 29 and

18 bp, respectively, and one insertion. The insertion mutation and one of the deletion mutations created a frameshift; the other deletion did not change the reading frame. Altogether, 15 different mutations were present in 22 out of 27 families with X-linked dominant CMTX1 as indicated in Table I. Figure 1 shows a diagram of the cx32 protein illustrating the locations of the mutations in the extracellular, intramembrane and cytoplasmic domains of the protein. The different mutations are spread throughout the protein with several being found in more than one family. For instance, The R→W mutation at amino acid 142 is located in the third transmembrane domain (families 9 and 10), the R → W mutation at position 164 is located in the second extracellular domain (families 11 and 20), and the R → E mutation at position 22 is located in the first transmembrane domain (families 2 and 18).

The impact of missense mutations on the phenotype was found to be variable. Three of the eight mutations produced a severe form of CMT, whereas the other five caused only mild forms of the disease. By contrast, the impact of all nonsense mutations, found at amino acids 22, 186, 217, and 220, produced a phenotype that was significantly more severe than that found with the missense mutations. Most patients showed severe weakness and atrophies of feet and hands. The 29 bp deletion, beginning at amino acid 265, produces a stop codon directly after amino acid 265. It also produces a severe CMT phenotype characterized by severe atrophies and weakness of feet and hands as well as proximal muscle involvement with early onset (at birth) in all four affected members. The other deletion of 18 bp is in frame and generates a protein in which amino acids 111 to 116 are deleted. The phenotype of this deletion is a mild to moderate CMT. The insertion of a G after the codon for amino acid 185 creates a shift in the reading frame resulting in a protein with an additional 56 random amino acids added after the threonine at position 185. This mutation also produces a severe CMT phenotype.

DISCUSSION

Previous papers [Ionasescu et al., 1994, 1995a] described five and 15 CMTX1 families, respectively, with point mutations in the coding region of the connexin 32 gene. The present study describes a total of 22 CMTX1 families with 15 different mutations of the same gene (Table I). Some of the point mutations of the cx32 gene had also been described by Bergoffen et al. [1993b] and Fairweather et al. [1994]. Most of the point mutations reported in this study, however, had not previously been reported. Our cases represent more than 50% of all the CMTX1 mutations that have been reported in the literature to date.

Attempts to explain the clinical CMT phenotype from the location and type of the cx32 mutation are hampered by our limited knowledge concerning the contribution of the various cx32 domains to cell-cell channel formation and their possible involvement in the regulation of channel functioning. The fact that most of the missense mutations produce only a mild CMT phenotype may be explained by the fact that the protein can tolerate amino acid changes at certain positions. This has been demonstrated previously by in vitro mutagenesis of cx32 cDNA and expression of the mutated cx32 mRNA in the oocyte expression system [Dahl et al., 1992; Werner et al., 1993]. It is surprising, however, that one missense mutation, the R → W change at

TABLE I. Connexin 32 Mutations (Iowa Findings)

Family	No. affected	Age of proband at examination	No. of years with symptoms	Phenotype[a]	Mutation	Substitution	Amino acid no.
1	13	41	29	Severe	C to T	Arg to stop	22
2	7	60	50	Moderate to severe	G to A	Arg to Gln	22
3	8	52	30	Mild	C to G	Trp to Ser	77
4	2	42	30	Mild	A to G	Gln to Arg	80
5	8	80	60	Mild	A to G	Glu to Gly	102
6	2	43	20	Mild	A to G	Glu to Gly	102
7	3	35	15	Mild	A to G	Glu to Gly	102
8	9	33	15	Mild to moderate	Del. of 18 bp	N.A.[b]	111–116
9	15	22	7	Mild	C to T	Arg to Trp	142
10	3	32	14	Mild	C to T	Arg to Trp	142
11	12	64	60	Moderate to severe	C to T	Arg to Trp	164
12	5	46	25	Severe	G insertion	N.A.	185
13	13	27	17	Severe	G to T	Glu to stop	186
14	19	39	37	Severe	T to A	Cys to stop	217
15	15	16	3	Severe	T to A	Cys to stop	217
16	5	43	19	Severe	C to T	Arg to stop	220
17	4	35	35	Severe	Del. of 29 bp	N.A.[b]	265–273
18	5	40	22	Moderate to severe	G to A	Arg to Gln	22
19	3	35	19	Moderate to severe	G to C	Trp to Ser	3
20	3	41	26	Moderate to severe	C to T	Arg to Trp	164
21	5	35	20	Mild	A to G	Glu to Gly	102
22	4	30	12	Mild	T to C	Trp to Arg	3

[a] Females have milder symptoms and later onset.
[b] N.A., not applicable.

position 142, that gave only a mild CMT phenotype destroyed functional channel formation in the oocyte expression system [Bruzzone et al., 1994]. Apparently, the oocyte assay system is more stringent in its requirement for the correct amino acid than the Schwann cell.

The three missense mutations that produce a moderate to severe CMT phenotype are located in the amino terminus (aa 3), the first transmembrane fragment (aa 22) and the second extracellular domain (aa 164), respectively. There is little information on the function of the aminoterminus. Clearly, the W → S mutation (family 19) seems to knock out connexin 32 function while a W → R mutation (family 22) at the same position is tolerated. The severe phenotype of the other two mutations are more easily explained. The first transmembrane segment may not tolerate amino acid changes because it is likely to be involved in the formation of the channel pore, and amino acid 164, although depicted as extracellular in Figure 1, may not remain extracellular when a channel is formed between two adjacent cells but participates in forming the channel wall [Dahl et al., 1992]. Thus, mutations at these sites are expected to produce nonfunctional cell-cell channels leading to a more severe phenotype.

All nonsense mutations, including the deletion mutation after codon 265 that creates a stop codon, cause

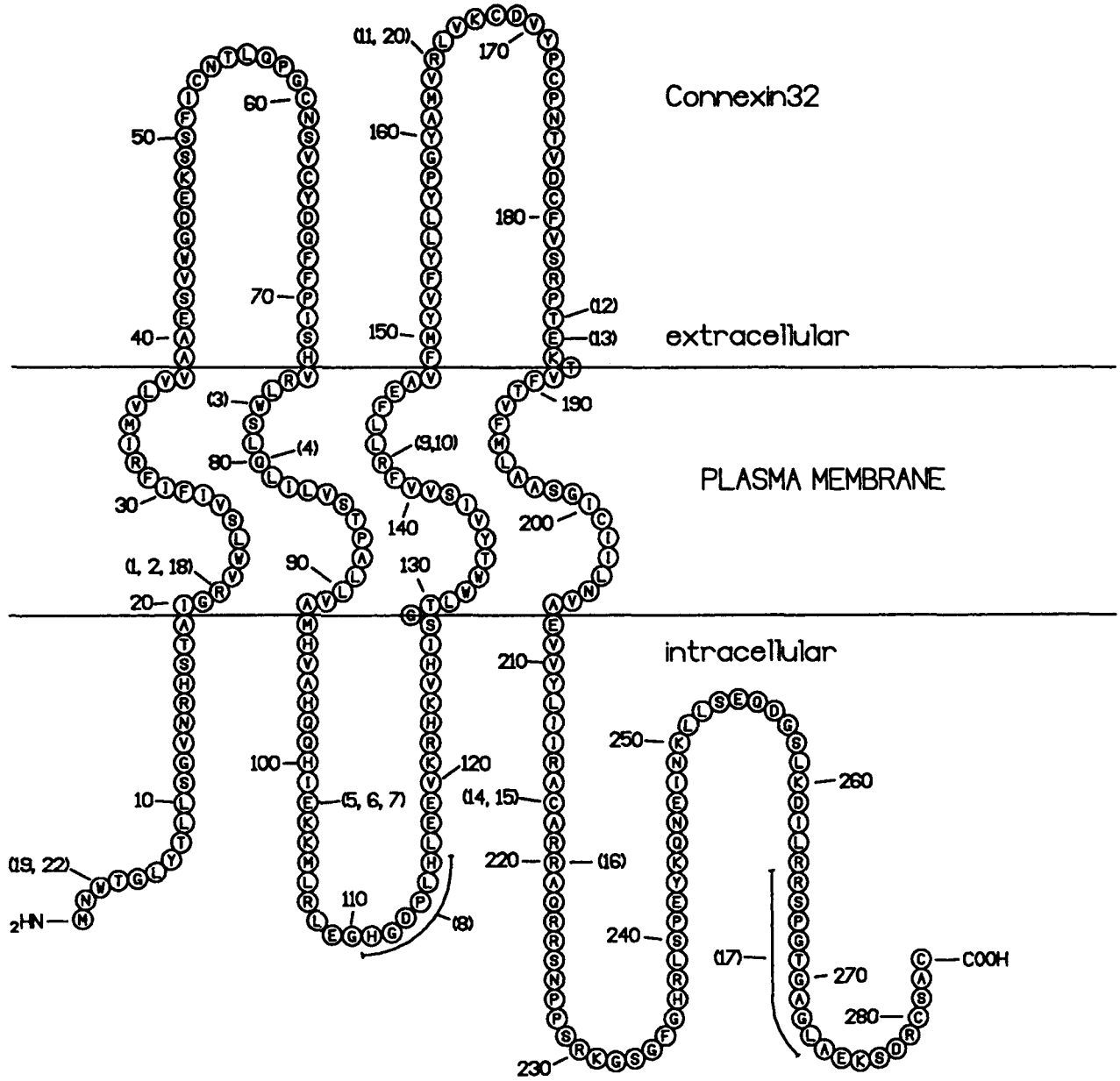

Fig. 1. Transmembrane topology of the human connexin 32. The locations of the identified mutations are indicated with the corresponding family numbers in parentheses.

severe CMT phenotypes. This is not unexpected because the proteins produced from such mutant genes would be truncated and thus are likely to be nonfunctional. It is interesting, however, that the nonsense mutation at amino acid 220, associated with a severe phenotype, still produces fully functional cell-cell channels when expressed in the oocyte system [Rabadan-Diehl et al., 1994]. Apparently, the carboxyterminus of the connexin 32 molecule plays an essential function in the Schwann cell that is not required for basic channel formation in the oocyte. The insertion of a G at position 185 creates a frameshift that causes the addition of 56 random amino acids at the site of the mutation. This mutation completely changes the fourth transmembrane region of the protein and thus is expected to be nonfunctional.

The in-frame deletion of six amino acids located within the cytoplasmic loop of the connexin molecule produces only a mild CMT phenotype. This suggests that the loop's main function is to connect two transmembrane segments of the protein. The shortening of the loop may interfere only slightly with the proper folding and membrane insertion of the connexin molecule thereby producing a mild CMT phenotype.

It is interesting that five additional CMT X-linked dominant families studied by us did not show mutations in the connexin32 coding region. Three families showed positive genetic linkage with the markers of the Xq13.1 region where the cx32 gene is mapped. Lod scores reached values between 3 and 7 at $\theta = 0$ in one family. The genetic linkage also showed positive lod scores in two other families but with lower values. Two families without point mutations had X-linked inheritance but their genetic linkage could not be established because of their small family size. We suggested that these cases may be caused by mutations in the connexin 32 promoter, splice sites untranslated regions. Further studies on these families are needed to clarify their molecular genetic etiology.

In conclusion, the severity of the CMT clinical phenotype seems to correlate with the location and type of mutation in the cx32 gene. Table I suggests that the childhood (early) onset of CMTX is more frequently associated with a severe phenotype than the classical adolescent phenotype. It seems that the duration of the disease is not as significant a factor for phenotype severity as the type of mutation. The families with the same type of mutation have similar severity rankings (Table I). The variability of the disease within a family seems to be related to the sex of the patient: affected males have a similar phenotype and progression. Affected females are always milder and with later onset than affected males. As more becomes known about the structure and function of the connexin32 protein, it is hoped that the results from this study will aid in the assignment of functions to the various regions of the connexin 32 protein.

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REFERENCES

Beckett J, Holden NE, Simpson BN, White BN, MacLeod PM (1986): Localization of X-linked dominant Charcot-Marie-Tooth disease to Xq13. J Neurogenet 3:225–231.

Bergoffen J, Trofatter J, Pericak-Vance MA, Haines JL, Chance PF, Fischbeck KH (1993a): Linkage localization of X-linked Charcot-Marie-Tooth disease. Am J Hum Genet 52:312–318.

Bergoffen J, Scherer SS, Wang S, Orozni Scott M, Bone LJ, Paul DL, Chen K, Lensch MW, Chance PF, Fischbeck KH (1993b): Connexin mutations in X-linked Charcot-Marie-Tooth disease. Science 262:2039–2042.

Bruzzone R, White TW, Scherer SS, Fischbeck KH, Paul DL (1994): Null mutations of connexin 32 in patients with X-linked Charcot-Marie-Tooth disease. Neuron 13:1253–1260.

Charcot JM, Marie P (1886): Sur une forme particulière d'atrophie musculaire progressive souvent familiale debutant par les pieds et les jambes et atteignant plus tard les mains. Rev Med 6:97–138.

Dahl G, Werner R, Levine E, Rabadan-Diehl C (1992): Mutational analysis of gap junction formation. Biophys J 62:187–195.

Dyck PJ, Lambert EH (1968): Lower motor and primary sensory neuron diseases with peroneal muscular atrophy. I. Neurologic, genetic and electrophysiologic findings in hereditary polyneuropathies. Arch Neurol 18:603–618.

Dyck PJ, Chance PF, Lebo RV, Carney JA (1993): Hereditary motor and sensory neuropathies. In Dyck PJ, Thomas PJ, Griffin JW, Low PA, Poduslo JF (eds): "Peripheral Neuropathy," 3rd edition. Philadelphia: Saunders, pp 1094–1136.

Fairweather N, Bell C, Cochrane S, Chelly J, Wang S, Mostacciuolo ML, Monaco AP, Haites NE (1994): Mutations in the connexin 32 gene in X-linked dominant Charcot-Marie-Tooth disease. Hum Mol Genet 3:29–34.

Fischbeck KH, Ar-Rushdi N, Pericak-Vance M, Rozear M, Roses AD, Fryns JP (1986): X-linked neuropathy: Gene localization with DNA probes. Ann Neurol 20:527–532.

Gal A, Mucke J, Theile H, Wieacker PF, Ropers HH, Wienker TF (1985): X-linked dominant Charcot-Marie-Tooth disease: Suggestion of linkage with a cloned DNA sequence from the proximal Xq. Hum Genet 70:38–42.

Goonewardena P, Welinhinda J, Anvret M, Gyftodimou J, Haegermark A, Iselius L, Linksten J, Petersson U (1988): A linkage study of the locus for X-linked Charcot-Marie-Tooth disease. Clin Genet 33:435–440.

Haites N, Fairweather N, Clark C, Kelly KF, Simpson S, Johnston AW (1989): Linkage in a family with X-linked Charcot-Marie-Tooth disease. Clin Genet 35:399–403.

Ionasescu V, Burns TL, Searby Ch, Ionasescu R (1988): X-linked dominant Charcot-Marie-Tooth neuropathy with 15 cases in a family: Genetic linkage study. Muscle Nerve 11:1154–1156.

Ionasescu V, Trofatter J, Haines JL, Ionasescu R, Searby Ch (1992): Mapping of the gene for X-linked dominant Charcot-Marie-Tooth neuropathy. Neurology 42:903–908.

Ionasescu V, Searby Ch, Ionasescu R (1994): Point mutations of the connexin 32 (GJB1) gene in X-linked dominant Charcot-Marie-Tooth neuropathy. Hum Mol Genet 2:355–358.

Ionasescu V (1995a): Charcot-Marie-Tooth neuropathies: From clinical description to molecular genetics. Muscle Nerve 18:267–275.

Ionasescu V, Searby Ch, Ionasescu R, Meschino W (1995b): New mutations and deletions of the connexin 32 gene in X-linked Charcot-Marie-Tooth neuropathy. Neuromusc Disord 5: 297–299.

Kunkel LM, Tantravahi V, Eisenhard M, Latt SA (1982): Regional localization on the human X of DNA segments cloned from flow sorted chromosomes. Nucl Acid Res 10:1557–1578.

Lathrop GM, Lalouel JM, Julier C, Ott J (1984): Strategies for multilocus linkage analysis in humans. Proc Natl Acad Sci USA 81:3443–3446.

Mostacciuolo NE, Miller E, Fardin P, Micaglio GF, Bardoni B, Guioli S, Camerino G, Danieli GA (1991): X-linked Charcot-Marie-Tooth

disease: A linkage study in a large family using 12 probes of the pericentromeric region. Hum Genet 87:23–27.

Pericak-Vance M, Barker DF, Bergoffen JA, Chance P, Cochrane S, Dahl N, Exier MC, Fain PR, Fairweather ND, Fischbeck K, Gal A, Haites N, Ionasescu R, Ionasescu V, Kennerson ML, Monaco AP, Mostacciuolo ML, Nicholson GA, Sillen A, Haines JL (1995):-Consortium fine localization of X-linked Charcot-Marie-Tooth disease (CMTX1): additional support that connexin 32 is the defect in CMTX1. Hum Hered 45:121–128.

Rabadan-Diehl C, Dahl G, Werner R (1994): A connexin 32 mutation associated with Charcot-Marie-Tooth disease does not affect channel formation in oocytes. FEBS Let 351:90–94.

Tooth HH (1886): "The Peroneal Type of Progressive Muscular Atrophy." London: H K Lewis & Co.

Werner R, Rabadan-Diehl C, Levine E, Dahl G (1993): "Affinities Between Connexins. Progress in Cell Research." New York: Elsevier Science, pp 21–24.